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Improved Solid Medium for the Detection and Enumeration of Pectolytic Bacteria

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An improved solid agar medium (MP medium) has been developed which allows detection of pectolytic activity in bacteria. Organisms tested exhibited a variety of regulatory controls governing pectate lyase synthesis. The medium contains mineral salts, pectin, and yeast extract. After growth of the organisms, the agar plate is flooded with a polysaccharide precipitant, and pectolytic activity is shown by clear zones around active colonies. High concentrations of phosphate are shown to be necessary for pectic enzyme formation on solid media. The medium has successfully been used to detect pectolytic organisms in soil, forest litter, and rotting vegetable samples.

The usefulness of a reliable medium on which bacteria capable of degrading pectin can be detected, enumerated, and isolated is without question. It is especially important that such a medium allow for rapid differentiation between organisms and that organisms subject to catabolite repression and other regulatory controls not be missed.

Several types of conditions and media have been used to detect organisms capable of producing soft rots and degrading pectin. One involves the use of sterile vegetable tissue, an example being the method of Kerr (7). Vegetable tissue is placed in a soil suspension for a brief time, removed, and incubated. Any soft rot of the tissue is then noted. Causative organisms may then be isolated from the rotted tissue.

The type of medium most commonly used relies on finding depressions or soft areas in a polypectate gel medium. The organisms growing on the gel cause the pectin to be dissolved (3, 4, 12, 13). A solid medium containing salicin has been described by Noble and Graham (11) for the isolation of *Erwinia* and *Aerobacter* sp. producing soft rots. When sodium pectate was used instead of salicin, growth of the organisms was unsatisfactory.

Each of the above media may be used to advantage for different conditions. The gel type of medium, however, requires considerable care in preparation. A solid medium may offer distinct advantages in both media preparation and detection and enumeration of organisms.

Recently Jayasankar and Graham (6) described a solid agar medium containing pectin and yeast extract. Pectolytic activity is determined after growth of the organisms by flooding the plate with cetyltrimethylammoniumbromide which precipitates intact polysaccharides. If the organism has degraded the pectin, a clear zone is seen around the colony against an otherwise opaque medium.

This technique appeared to offer certain advantages over other methods. However, in our hands, organisms known to be strongly pectolytic failed to degrade pectin on the Jayasankar and Graham medium. Methods to resolve these inadequacies in the medium as well as further means to provide high levels of pectolytic enzyme synthesis on solid media, in organisms exhibiting a variety of regulatory controls, are described in this report.

MATERIALS AND METHODS

Organisms used. *Erwinia carotovora* ATCC 8061, *E. carotovora* 1, *E. aroideae* 1, *E. atroseptica* 201, and *Pseudomonas marginalis* 2 were obtained from M. N. Schroth and D. C. Hildebrand, Dept. of Plant Pathology, Univ. of Calif., Berkeley; *Pseudomonas* sp. 62-27 is associated with onion rots and was obtained from R. Dickey, Dept. of Plant Pathology, Cornell Univ., Ithaca, N.Y.

Preparation of cultures for plating. All cultures were grown for 24 hr in a shaken culture at 30 C in mineral medium containing 0.1% of glucose. This bacterial suspension was diluted before plating on the various media described.

Preparation of potato extract. A 2.4-g amount of an acetone powder of potato prepared as previously described (15) was homogenized with 37.5 ml of mineral medium and 0.7 ml of 0.1 M $NaHSO_3$ in a Ten Broeck tissue grinder. The suspension was filtered through a Seitz filter and the clarified extract

was sterilized by filtration through a membrane filter (0.22 μm; Millipore Corp., Bedford, Mass.).

Polysaccharide precipitant and use. Plates were flooded with a 1% solution of hexadecyltrimethylammoniumbromide (J. T. Baker Chemical Co.) in water, taking care not to dislodge colonies. Zones of pectolysis were measured as soon as they appeared, usually within 15 min. The solution may be sterilized by autoclaving before use if desired.

Media. All media were sterilized by autoclaving (121 C, 15 min) except the potato extract. This extract, previously sterilized by membrane filtration, was added to the agar medium which had been tempered to 45 C.

JG medium, described by Jayasankar and Graham (6) contained, per liter: K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; NaCl, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.2 g; $FeCl_3 \cdot 6H_2O$, 0.01 g; yeast extract, 1 g; apple pectin, 5 g; agar, 20 g; *p*H 7.0. The original medium calls for 20 g of agar per liter, but we have used 15 g satisfactorily. When potato extract was added to this medium, two parts of sterile medium (2 X concentrated) were added to one part sterile potato extract and one part sterile water. The yeast extract was omitted. Mineral medium (14) contained, per liter: $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 4 g; Na_2HPO_4, 6 g; $FeSO_4 \cdot 7H_2O$, 1 mg; $MgSO_4$, 0.2 g; $CaCl_2$, 1 mg; H_3BO_3, 10 μg; $MnSO_4$, 10 μg; $ZnSO_4$, 70 μg; $CuSO_4$, 50 μg; MoO_3, 10 μg; *p*H 7.4. For mineral medium containing agar and pectin, pectin (Apple, Schwarz-Mann, Orangeburg, N.Y.) was added to the above described mineral medium at the rate of 5 g per liter and agar at 15 g per liter. Mineral medium containing pectin and either yeast extract or potato extract was made by preparing mineral medium with pectin at 2X concentration. An equal volume of sterile yeast extract (0.2% in water) was added to give single-strength concentration. When potato extract was used, one part of it (sterile) was added to two parts 2X mineral medium with pectin and one part water to provide single-strength concentration.

RESULTS AND DISCUSSION

Four representative soft-rot pathogens were tested on the medium of Jayasankar and Graham (6), hereafter called JG medium. At the same time, the JG medium was supplemented with potato extract instead of yeast extract. Zucker and Hankin showed that potato tissue (15) or potato extract (*unpublished data*) added to a medium containing pectin exerts a synergistic effect on induction of pectate lyase, a major pectolytic enzyme in some soft-rot pathogens. The potato extract is prepared in mineral medium, and thus mineral medium alone was added to the JG medium as a control (Table 1).

The most striking outcome was that pectolytic activity was observed only on the plates containing mineral medium. The JG medium itself failed to show any pectolytic activity with the known soft-rot organisms tested. Further, where pectolytic activity occurred, it was observed only in the surface colonies; this is reflected in the percentage of colonies showing pectolytic activity in Table 1. Subsurface colonies were nonpectolytic, suggesting that pectolytic enzyme synthesis requires aerobic conditions. Based on these observations, all subsequent experiments were made by a spread plate technique instead of by pour plate as recommended by Jayasankar and Graham (6).

Experiments were conducted to ascertain why the JG medium failed to provide the conditions suitable for synthesis of pectolytic enzymes. Additions of mineral medium to the JG medium (Table 1) overcame this problem. Generally, the mineral medium is high in phosphate and low in calcium, JG medium being the opposite. Consequently, JG medium to which phosphate had

TABLE 1. *Pectolytic activity and growth of some phytopathogenic bacteria on three media*[a]

Organism	Activity on media						
	JG		JG (no yeast extract) + potato extract		JG + mineral medium		Plate count agar (no. of colonies)
	No. of colonies[b]	Per cent pectolytic[c]	No. of colonies	Per cent pectolytic	No. of colonies	Per cent pectolytic	
Erwinia aroideae	57	0	175	31	198	22	218
Pseudomonas marginalis	175	0	248	42	229	29	188
Pseudomonas sp. 62–27	197	0	242	12	236	31	252
E. carotovora 8061	175	0	226	21	76	45	138

[a] JG, medium of Jayasankar and Graham (6). The potato extract is prepared in mineral medium; two parts JG medium (2X concentrated) to which one part mineral medium is added served as control.

[b] Pour plates were used, and incubation was for 72 hr at 30 C. The same dilution series was used for each organism. Counts between organisms are not comparable, only counts between media.

[c] Represents per cent of colonies showing zones of pectolytic activity. Since only the surface colonies (or just subsurface) showed activity, this number represents colonies in that position in the agar.

been added, or from which calcium was removed, was tested (Table 2). Our observations indicate that a higher level of phosphate than that in JG medium is needed to promote pectolytic enzyme synthesis. The stimulatory effect of phosphate on pectate lyase production has been shown by Hsu and Vaughn (5) with *Aeromonas liquefaciens*. In the view of the foregoing data, it was determined that a medium composed of mineral medium containing 0.5% pectin and 0.1% yeast extract was most suitable for the detection and enumeration of pectolytic organisms. This general medium has been designated as MP medium.

Since potato tissue and potato extract are known to contain a synergistic factor for induction of pectate lyase in some organisms (15; *unpublished data*), a series of bacterial phytopathogens was tested on mineral medium containing pectin and either yeast extract or potato extract (Table 3). In this table, the size of the zone of pectolytic activity and the ratio of colony size to zone size are shown. Colony size for each organism was similar on both media. In general, zones were larger on potato extract medium. However, the mineral medium with pectin and yeast extract is advocated for general use rather than potato extract since it is easier to prepare. Potato factor is heat-labile (15) and requires more care to prepare and use.

Pectolytic organisms produce two major types of pectolytic enzymes, pectate lyases (EC 4.2.99.3) and polygalacturonases (EC 3.2.1.15; reference 2). Lyases are usually characterized by alkaline *p*H optima, whereas polygalacturonases have more acid optima. An attempt was made by adjusting the *p*H of the medium to differentiate between lyase and polygalacturonase activity on MP agar plates. Table 4 shows the results obtained with three bacterial phytopathogens. The organisms grew well at all *p*H levels but showed most pectolytic activity at *p*H 8, indicating strong pectate lyase synthesis. As the *p*H of the medium decreased, less pectolytic activity was noted. At *p*H 5, only *P. marginalis* showed any pectolytic activity. The high activity at *p*H 8 and the lack of, or decrease in pectolytic activity, at *p*H 5 suggest that pectate lyases are the major pectolytic enzymes produced by the test organisms on solid media. Similar results have been obtained in liquid culture (8, 9).

To test whether any lyase was synthesized at the more acid *p*H levels, both *P. marginalis* and *E. carotovora* were inoculated into liquid media ranging in *p*H from 5 to 8. After 24 hr of growth, extracellular lyase activity was measured at an optimal *p*H of 8.8 by a spectrophotometric assay at 235 nm (1). Table 5 indicates no lyase forma-

TABLE 2. *Phosphate requirement for pectolytic activity of some phytopathogens*

Media	Pectolytic activity[a]		
	Pseudomonas marginalis	*Erwinia carotovora* (8061)	*E. aroideae*
JG medium	−[b]	−	−
Plus phosphate[c]	+	+	NT[d]
Minus Ca[e]	−[b]	−	−
Mineral medium with pectin and yeast extract	+	+	+

[a] Pectolytic activity shown as − or +: +, indicates all colonies showed pectolytic actvitiy; −, none showing activity. Spread plate technique was used; incubation was at 30 C for 72 hr.

[b] Some very faint zones (small and barely visible) of pectolytic activity seen with a few colonies.

[c] Medium of Jayasankar and Graham (6) to which KH_2PO_4 and Na_2HPO_4 were added at concentrations found in mineral medium.

[d] Not tested.

[e] JG medium with $CaCl_2$ level at 1 mg per liter instead of 200 mg per liter.

TABLE 3. *Comparison of yeast extract and potato extract in mineral medium containing pectin for determination of pectolytic activity of some phytopathogens*[a]

Organism	Results with yeast extract			Results with potato extract		
	No. of colonies	Avg zone size (cm)	Ratio (colony to zone size)	No. of colonies	Avg zone size (cm)	Ratio (colony to zone size)
Erwinia atroseptica	85	0.7	1.4	120	1.3	3.3
Erwinia aroideae	65	0.7	1.5	40	1.0	2.5
Erwinia carotovora (8061)	30	0.4	2.0	10	0.9	3.6
Erwinia carotovora (# 1)	190	0.7	1.8	75	1.2	2.2
Pseudomonas marginalis	122	0.9	3.3	210	1.1	3.4
Pseudomonas sp. 62-27	130	0.8	3.8	180	1.1	4.2

[a] Spread plate technique was used; incubation was at 30 C for 48 hr.

tion by either organism at *p*H 5, but *E. carotovora* did produce some active enzyme at *p*H 6. Since *P. marginalis* produced no lyase when grown at *p*H 5, its pectolytic activity at this *p*H (Table 4), may thus be attributed to polygalacturonase synthesis.

The pectolytic medium (MP medium) as described above has been tested with soil, forest litter, and samples of rotting vegetable materials. By using a spread plate technique, pectolytic organisms have been found in all five soils examined and in six different rotting vegetables including spinach, carrots, and cauliflower. The soil samples were tested by sprinkling a few milligrams of soil directly on the surface of the agar or by plating a dilution series.

The mineral medium containing pectin and yeast extract may be used to detect a wide variety of pectolytic bacteria. However, the use of more specialized media containing inhibitors for different groups of organisms should not be overlooked. They provide extremely useful tools in screening for plant pathogens or pectolytic soil organisms. All pectolytic organisms are not aerobes (10), and the proposed media could, of course, be used under anaerobic culture conditions.

The usefulness of the proposed medium is not limited to isolation of potential pathogens. It has an application important in the current emphasis on biodegradation of various types of waste materials. A proposal has been made to dispose of waste by plowing garbage into the upper layers of agricultural soils. In this connection it is important to choose an area with soil rich in pectolytic organisms, and the mineral medium with pectin and yeast extract is especially useful in enumerating such organisms.

TABLE 4. *Pectolytic activity of three bacterial phytopathogens on mineral medium containing pectin and potato extract adjusted to different pH values*[a]

Organism	Pectolytic activity at			
	*p*H 5	*p*H 6	*p*H 7[b]	*p*H 8
Erwinia carotovora (8061)	0	1	8	10
Pseudomonas marginalis	2	4	8	10
E. aroideae	0	1	8	10

[a] Pectolytic activity rated on a 0 to 10 scale, the higher number denoting strong pectinolysis. Spread plate technique was used; incubation was at 30 C for 48 hr.

[b] Citrus pectin (Pectin, N. F., Sunkist Growers, Corona, Calif.) was also used at *p*H 7 with similar results.

TABLE 5. *Production of pectate lyase by Erwinia carotovora ATCC 8061 and Pseudomonas marginalis in shaken culture (30 C) for 24 hr at various pH values*

*p*H	Activity of *E. carotovora*[a]		Activity of *P. marginalis*[a]	
	Growth[b]	Pectate lyase activity[c]	Growth[b]	Pectate lyase activity[c]
5	0.3	<0.1	0.1	<0.1
6	0.7	0.9	0.3	<0.1
7	1.2	8.0	0.2	6.6
8	1.2	18.5		

[a] *E. carotovora* was grown in mineral medium containing pectin and potato extract, and *P. marginalis* was grown in mineral medium containing 0.1% of glucose.

[b] Values expressed as 10^{10} cells per ml.

[c] Values expressed as units per 10^{10} cells. One unit of pectate lyase catalyzes the formation of 1 μmole of unsaturated uronide per minute from sodium polypectate under conditions of assay (15) at *p*H 8.8. Cell number was measured turbidimetrically.

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